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Please provide the following references:

1) J Chromatogr 1990 Feb 23;525(2):297-306
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Thank you,
David J. Steadman
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Rapid Isolation of High Molecular Weight Urokinase from Nativ Human Urine

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Key words

Urokinase – HMW-urokinase – Native urine – Plasminogen activator – Activity – Gelatine-Sepharose

Summary

Urokinase (UK) could be purified to apparent homogeneity starting from crude urine by sequential adsorption and elution of the enzyme to gelatine-Sepharose and agmatine-Sepharose followed by gel filtration on Sephadex G-150. The purified product exhibited characteristics of the high molecular weight urokinase (HMW-UK) but did contain two distinct entities, one of which exhibited a two chain structure as reported for the HMW-UK while the other one exhibited an apparent single chain structure. The purification described is rapid and simple and results in an enzyme with probably no major alterations. Yields are high enough to obtain purified enzymes for characterization of UK from individual donors.

Introduction

The fibrinolytic activity of urine uncovered by its ability to dissolve fibrin clots in 1885 by Sahli (1) and attributed to a urine activator of plasminogen, urokinase (UK, E.C. 3.4.21.31) by Sobel in 1952 (2), could be purified to apparent homogeneity using conventional purification procedures (3–8), taking advantage of the high affinity of UK to surfaces (9, 10) or applying the technique of specific affinity chromatography (11–14). However, the latter procedure has been applied only to commercially available prepurified material, because the use of crude urine as starting material resulted in rather weak binding of the enzyme to the affinity matrix and increased activity losses (14). The complicated and time consuming conventional procedures on the one hand and the use of commercially available urokinases as starting material on the other hand resulted in low yields and in a molecular weight (M_r) distribution of UK different from that in native urine; besides a 54.000 molecular weight form predominantly present in native urine (high molecular weight UK, HMW-UK, S_2-type) (7, 8, 10, 11), a 31.000 molecular weight form (low molecular weight UK, LMW-UK, S_1-type) (8–11) could be purified to homogeneity. Furthermore, a plasminogen activator with a M_r of about 100,000 could be detected in native urine (15, 16). Based on all these observations and the fact that both purified forms of UK are two polypeptide chain enzymes (17), the question about the native form of UK has been raised. It was the aim of this study to develop a purification scheme for rapid isolation of UK starting from native human urine in order to minimize alterations of the molecule and with yields high enough for characterization of the enzyme.

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Materials and Methods

Cyanogen bromide (Fluka AG, Switzerland), Triton X-100 and Tween 80 (Merck-Schuchardt, GFR), Aqua-Sil (Pierce, USA), human serum albumin, transferrin, ovalbumin, chymotrypsinogen A (Pharmacia, Sweden), agmatine sulfate (Sigma, USA), Sephadex G-150 and Sepharose 4-B (Pharmacia, Sweden), acrylamide, bisacrylamide, sodium-dodecyl-sulfate (BioRad, USA), diisopropylfluorophosphate (1-^{3}H[N]) 1.00108 Ci/mmol (DFP, New England Nuclear, USA), Spectrapor membrane tubing No. 1, cut off, M_r = 6000–8000 (Spectrum Med. Ind. Inc., USA), gelatine (Becton Dickinson Laboratories, USA), bovine plasminogen-containing and plasminogen-free fibrinogen (Poviet, The Netherlands), pyro-Glu-Gly-Arg-pNA (S-2444) and H-D-Val-Leu-Lys-pNA (S-2251) (Kabi, Schweden), antibodies against chicken egg-albumin and human transferrin (Behringwerke, GFR), iodoacetamide (Calbiochem, USA), and 2-mercaptoethanol (Merck, GFR), were obtained as indicated.

Preparation of Substituted Sepharose 4-B Gels

Sepharose 4-B was activated by addition of solid cyanogen bromide (15 gr/100 ml gel, 50% v/v in destilled water) (18). For the preparation of gelatine substituted Sepharose 4-B 400 mg of gelatine dissolved in 200 ml of 0.1 M $NaHCO_3$ buffer containing 1 M NaCl (pH 8.9) were added to 75 ml Sepharose 4-B (settled volume). After 12 hr end-over-end rotation at room temperature the gel was washed and equilibrated in 0.01 M Tris.HCl buffer (pH 7.4). The procedure resulted in the coupling of 0.8 mg of gelatine to 1 ml activated Sepharose 4-B (settled volume). EACA-agmatine-Sepharose 4-B (13) and lysine-Sepharose (19) were prepared essentially as described.

For monitoring plasminogen activator activity (PAA) during the purification procedure plasminogen-rich fibrin agarose plates were used (20). To determine yields and specific activities in the starting material, after each purification step and in the final product, amidolytic activities on S-2444 (21) and fibrinolytic activities using a fibrin clot assay (22) were evaluated. For standardization of the plasminogen activator assays UK-Serono was used and PAA was expressed in CTA units per ml.

Plasminogen used for kinetic studies was prepared from human plasma by affinity chromatography on lysine-Sepharose (19) followed by gel filtration on Sephadex G-150.

Protein was determined using the dye reagent and standards from BioRad (23); protein for the elution profiles was monitored at 280 nm.

To evaluate the molecular weight distribution of PAA during the purification, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on slab gels (24) using 7.5% polyacrylamide gels. Samples were run in duplicate and after electrophoresis the slab gels were cut lengthwise. One segment was fixed and stained (25), the other segment was washed in 2% Triton X-100 for 1 hr and afterwards layered on top of plasminogen-rich fibrin agarose plates. Areas of lysis were determined after 8 hr of incubation at 37° C.

Polyacrylamide gel isoelectric focusing (PAGIF) of the purified material was performed in a flat bed apparatus (FBE 3000, Pharmacia, Sweden) using a 4.85% polyacrylamide gel containing 6% glycerol and 2.5% pharmalyte, pH = 6.5–10 (Pharmacia, Sweden). After prefocusing for about 500 volt hr, 0.1 ml of the sample was applied to the middle of the gel and focusing was continued for 90 min resulting in a total of about 4000 volt hr. After focusing, the pH gradient was measured in 5 mm intervals using an Ingold surface electrode (Ingold, Switzerland) connected to a Beckman digital pH-meter. The samples were applied in duplicate and after focusing one gel segment was cut in 5 mm pieces,

eluted with 0.05 M Tris.HCl buffer (pH 7.5) and tested for PAA on plasmin-rich fibrin agarose plates; the replica gel was fixed and stained (25).

Alkaline disc electrophoresis was carried out at pH = 8.2 (26). Samples were run on replica gels. One gel was cut into 2 mm slices, eluted in 0.05 M Tris.HCl buffer (pH 7.5) and tested for PAA on plasmin-rich fibrin agarose plates, the other gel was fixed and stained as above (25).

Sucrose density gradient centrifugation of purified UK preincubated for 30 min at 37° C with or without 0.2% gelatine (final concentration) was performed in 4 ml cellulose nitrate tubes (Beckman, USA) in 5–20% sucrose gradients in 0.01 M Tris.HCl buffer (pH 7.4) with or without addition of 0.5 M $CaCl_2$. After centrifugation at 60.000 rpm for 2 hr at 4° C in a vertical rotor (Sorvall TV-865 ultra vertical rotor, DuPont, USA) in a preparative ultracentrifuge (Sorvall OTD 2 ultracentrifuge, DuPont, USA) fractions of 0.3 ml were collected. Each fraction was tested for its refractive index using a Zeiss-Abbé-refractometer (Zeiss, GFR) and for its PAA on plasmin rich fibrin agarose plates. Transferrin and ovalbumin were centrifuged in separate tubes in the same run and their locations in the gradient were determined by counter current electrophoresis against their respective antisera (27).

Isolation Procedures

All procedures were performed at 4° C; all columns, glass wares and tubes used were siliconized (28), all buffers used in the three purification steps contained 0.1% Tween 80 to minimize adsorption of the enzyme to surfaces (12). In each purification 1300 to 2100 ml of crude human urine obtained from healthy male laboratory personnel were used as starting material. After collection, the urine was centrifuged at 13.000 × g for thirty min. The supernatant was made 0.02% in gelatine and dialyzed against several changes of 0.01 M Tris.HCl buffer (pH 7.4), until conductivity had dropped below 1 mSiem.

Chromatography on gelatine-Sepharose (step I). To 1300–2000 ml of dialyzed crude urine 70 ml of gelatine-Sepharose were added and rotated end-over-end for 3 hr. Thereafter, the gel was collected and washed on a Buchner funnel with 0.01 M Tris.HCl buffer (pH 7.4) and filled into a 13.5× 2.6 cm column; the column was developed with a gradient from 0 M to 0.7 M $CaCl_2$ in 0.01 M Tris.HCl buffer (pH 7.4) at a flow rate of 26.5 ml/hr and 3.1 ml fractions were collected. Fractions containing PAA were pooled, made 0.02% in gelatine and 10^{-3} M in EDTA and dialyzed against 0.01 M potassium phosphate buffer (pH 7.4) containing 0.02% gelatine and 10^{-3} M EDTA until conductivity had dropped below 2 mSiem.

Affinity chromatography on agmatine-Sepharose (step II). To the pooled and dialyzed material obtained from step I 30 ml settled volume of agmatine-Sepharose were added and rotated end-over-end for 15 hr. Thereafter, the gel was collected and washed on a Buchner funnel with 0.02 M potassium phosphate buffer (pH 7.4) and filled into a 1.6 × 15 cm column; the column was eluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.4 M KCl. Fractions of 3.1 ml were collected at a flow rate of 23 ml/hr. Fractions containing PAA were pooled, freeze-dried and reconstituted with 10 ml of destilled water.

Gel filtration on Sephadex G-150 (step III). Five ml of the reconstituted material obtained after step II were gel filtrated on a Sephadex G-150 column (100 × 2.6 cm), equilibrated in 0.1 M Tris.HCl buffer (pH 7.4) containing 1.0 M NaCl at a flow rate of 11 ml/hr. Fractions containing PAA were pooled, concentrated by dialysis against dry Sephadex G-200 and used as purified HMW-urokinase.

Enzyme Kinetic Studies with S-2444 as a Substrate

Amidolytic activity of the purified HMW-UK was determined using S-2444 as a substrate. For determination of enzyme kinetics the purified HMW-UK (7.6, 3.8, 1.9 CTA units final concentration) was incubated with 4 different substrate concentrations (600, 300, 150, and 75 µM) for up to 3 hr at 37° C. The cleavage of the substrate was monitored at 405 nm and expressed in ΔA/min; using the molar extinction coefficient for paranitroanilide, E_{405} = 10,500 (29), the moles of substrate cleaved per minute could be calculated. In the same assay system the effects of gelatine (0.05% and 0.5% final concentration), and $CaCl_2$ (0.1 M and 0.7 M final concentration) on the amidolytic activity of UK were studied.

Enzyme Kinetic Studies with Plasminogen as a Substrate

To determine PAA of the purified UK, 3 different concentrations of UK (11.5, 7.6, 5.1 CTA units final concentration) were incubated with plasminogen at 1.11, 0.55, 0.27, and 0.13 uM substrate concentration. To quantitate the formed plasmin, S-2251 was used in a concentration of $5.45 \cdot 10^{-4}$ M (29, 30) and cleavage of the substrate was monitored at 405 nm. From the increase in A at 405 nm per minute the amount of plasmin present in the incubation mixture could be calculated for different time points (A_{405}/min per nM plasmin = 0.01). Plasmin formed per min was used for calculation of the kinetics of plasminogen activation (29).

^{3}H-DFP-Incorporation

One hundred µl of purified UK were incubated with 890 µl of 0.1 M Tris.HCl buffer (pH 8.0) and 10 µl of ^{3}H-DFP (10 nM final concentration) for 12 hr at room temperature. For control purposes the purified UK was treated in the same way without addition of ^{3}H-DFP. After extensive dialysis against several changes of 0.1 M Tris.HCl buffer (pH 8.0) the remaining enzyme activities in the ^{3}H-DFP treated and in the untreated UK samples as well as the radioactivity of the ^{3}H-DFP treated preparation were determined (Beckman liquid scintillation counter LS 7500). The ^{3}H-DFP treated UK was used for the determination of the chain structure by means of SDS-PAGE of the reduced and unreduced sample.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of the Reduced and Unreduced HMW-UK

SDS-PAGE of ^{3}H-DFP-labelled UK was performed in 10% polyacrylamide gels in 0.1 M phosphate buffer (pH 7.1) containing 0.1% SDS (25). Samples were either treated for 2 hr at 37° C with mercaptoethanol (1% final concentration) followed by 30 min of alkylation with iodoacetamide (0.05 M final concentration), or were only alkylated. After electrophoresis at 8 mA per gel the gels were cut into 2 mm slices, eluted in 1 ml of 0.05 M Tris.HCl buffer (pH 7.0) and tested for radioactivity in a liquid scintillation counter (Beckman liquid scintillation counter LS 7500). IgG, albumin, transferrin and chymotrypsinogen A were used as molecular weight markers, treated as the samples and run in separate gels at the same time.

Results

The results of the chromatography of dialyzed crude urine on gelatine-Sepharose are shown in Fig 1. PAA of the dialyzed material represented about 30% of the activities originally present in the collected urine. More than 90% of the PAA

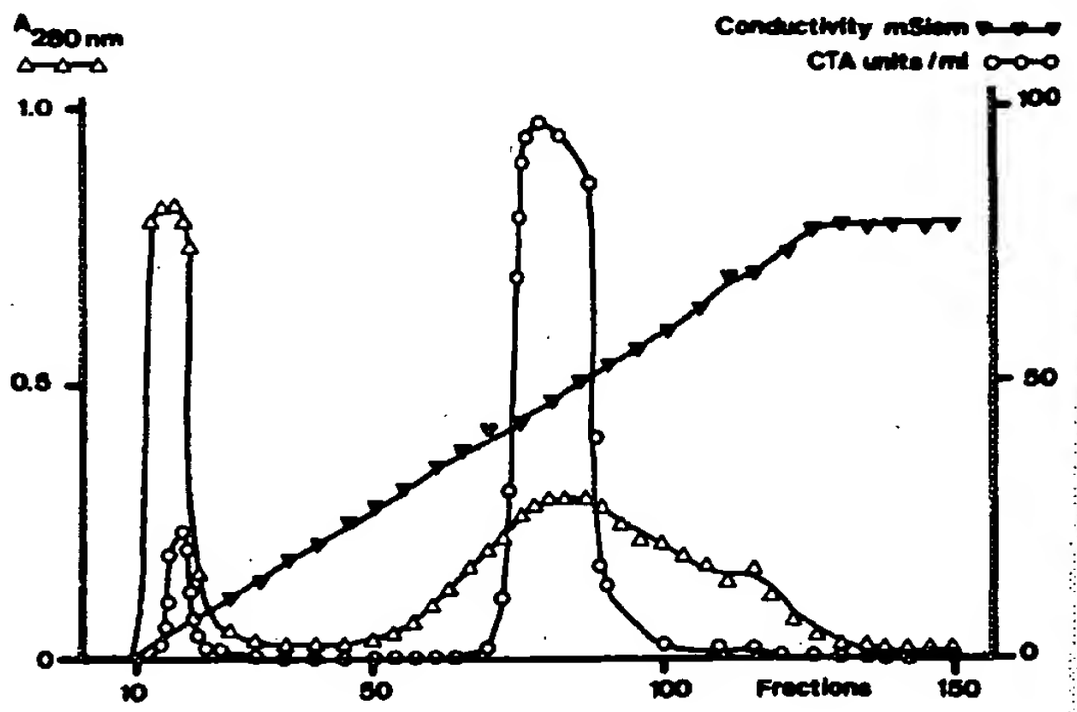

Fig. 1 Chromatography of dialyzed crude urine on gelatine-Sepharose. Elution with 0 M to 0.7 M $CaCl_2$ gradient in 0.01 M Tris.HCl buffer (pH 7.4). Gradient was started at fraction 1. Fractions 72 to 92 were pooled and used for step II.

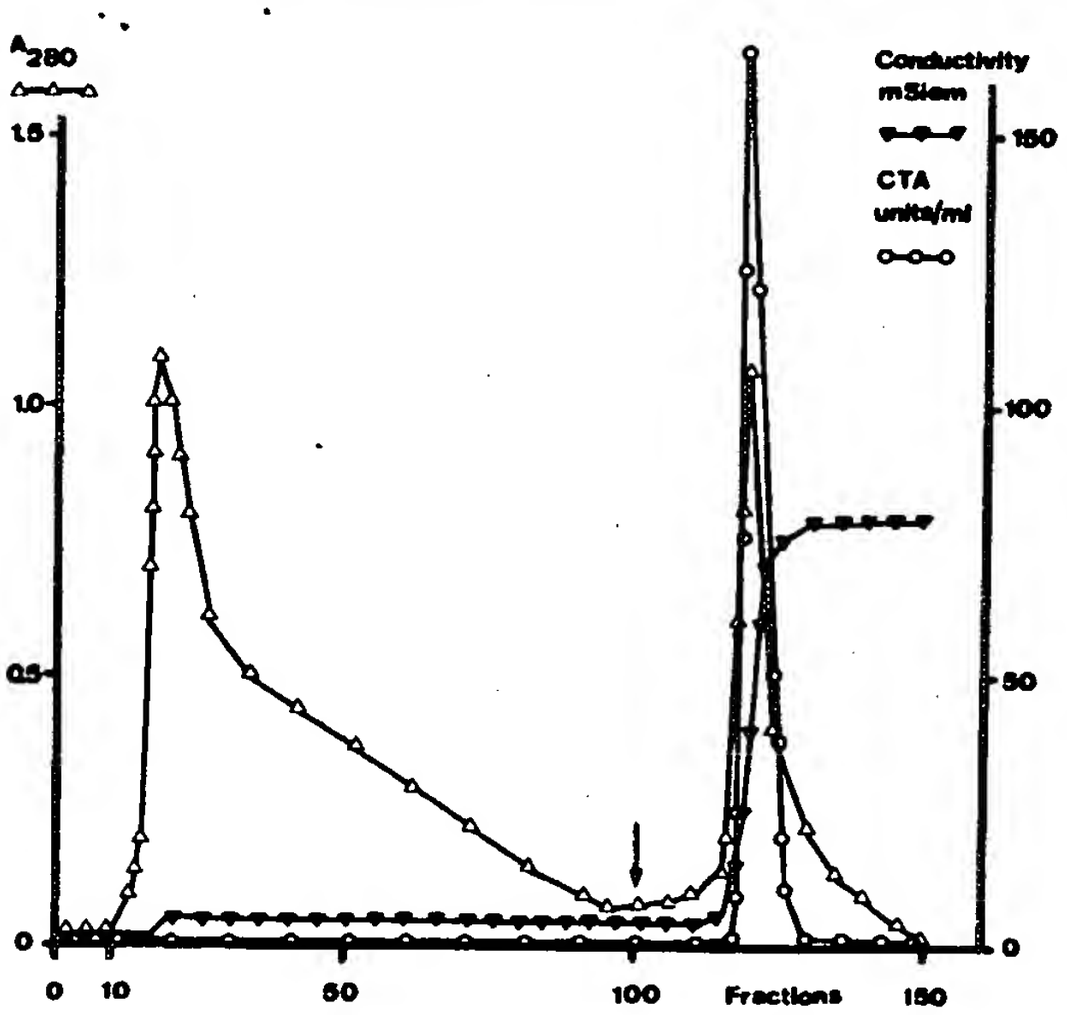

Fig. 2 Chromatography of dialyzed crude urine on gelatine-Sepharose. The column was first developed with 0.01 M Tris.HCl buffer (pH 7.4) containing 0.02 M $CaCl_2$ and from fraction 100 on with 0.01 M Tris.HCl buffer (pH 7.4) containing 0.7 M $CaCl_2$. Fractions 117 to 130 were pooled and used for step II.

applied to the gelatine-Sepharose were bound, while most of the protein appeared in the non bound fraction. Immediately after the elution gradient was started, a small peak of PAA could be eluted associated with a large protein peak, while the main PAA was eluted as a single peak in the middle of the gradient at about 40 to 50 mSiem, representing 70% of the activities originally applied. This peak of activity was associated with a flat protein elution peak.

After 3 experiments the gradient elution was substituted by a step elution using 0.01 M Tris.HCl buffer (pH 7.4) containing 0.02 M $CaCl_2$ as a first step followed by 0.01 M Tris.HCl buffer (pH 7.4) containing 0.7 M $CaCl_2$ (Fig 2). With the first step a broad protein peak associated with only trace amounts of activities could be eluted, while the second step resulted in the elution of more than 70% of the activities associated with only small amounts of protein.

Figure 3 shows the elution of PAA from the agmatine-Sepharose column. While the bulk of proteins was not bound to the agmatine-Sepharose and appeared in the fall through, 100% of UK was bound. With increasing the ionic strength a single peak of activity and only small amounts of protein could be eluted which represented more than 80% of the PAA originally applied to the gel.

Figure 4 shows the gel filtration of the material obtained after step II on Sephadex G-150. Most of the protein was excluded from the column and appeared as peaks in the void volume and slightly behind; the latter one was associated with small amounts of PAA. PAA was mostly included and could be eluted together with trace amounts of protein at an elution volume corresponding to a M_r of 56,000. The HMW-UK represented more than 80% of the activities originally applied to the column.

The total recovery of enzyme activity for the whole purification procedure was 10.5% and an about 4,000-fold purification could be calculated over the starting material. Purification and yields are summarized in Table 1 as means and standard errors of the means (S.E.) for 6 experiments. The final product could be shown to be homogenous by alkaline disc electrophoresis (Fig 5). A faintly staining band could be seen at the cathodic end of the gel corresponding to the region where PAA could be eluted. Isoelectric focusing of the purified material revealed a pH region between 8.7 and 9.05 where PAA could be detected with a peak of activity at pH=8.9. The purified material subjected to SDS-PAGE on slab gels exhibited PAA and stainable protein corresponding to a M_r of 54,000. SDS-PAGE of the ^{3}H-DFP treated material resulted in elutable radioactivity from the unreduced and alkylated sample exclusively corresponding to a M_r of 54,000. The reduced and alkylated sample showed radioactivity corresponding to M_r of 54,000 (60%) and 31,000 (40%), respectively.

The material characterized as above was used as purified HMW-UK in the kinetic experiments. Analysis of the cleavage of S-2444 at different enzyme and substrate concentrations by Lineweaver-Burk double reciprocal plot revealed one common point of intersection on the abscissa at a K_m value of

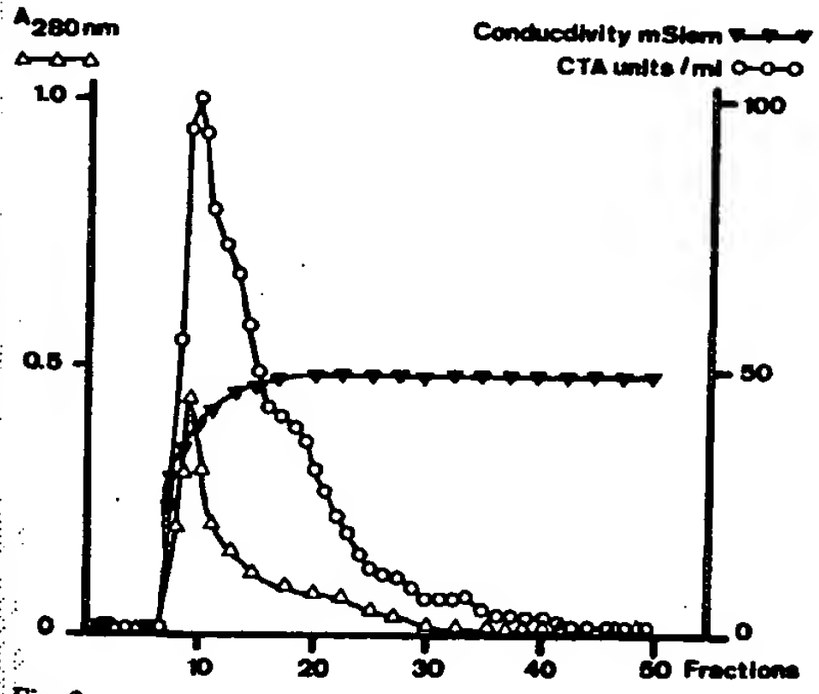

Fig. 3

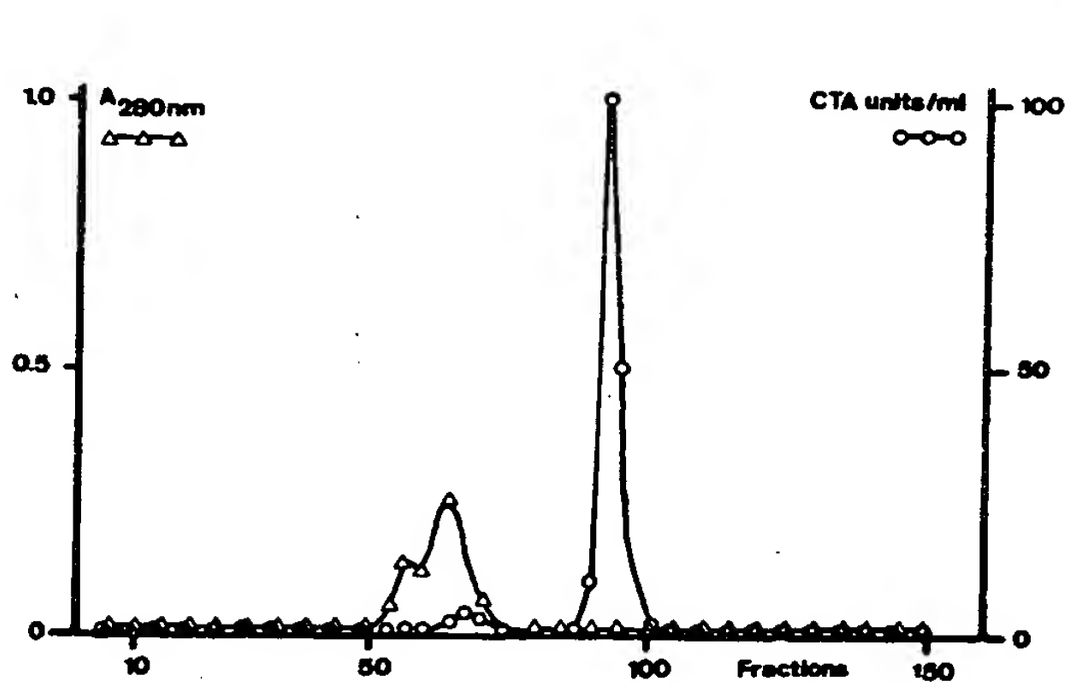

Fig. 4

Fig. 3 Chromatography of the material obtained after step I on EACA-agmatine-Sepharose. Elution was started at fraction 1 with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.4 M KCl. Fractions 7–26 were pooled and used for further purification.

Fig. 4 Gel filtration of the dialzyed and concentrated material obtained after step II on Sephadex G-150 in 0.1 M Tris.HCl buffer (pH 7.4) containing 1.0 M NaCl. Fractions 90 to 100 were pooled, concentrated by dialysis against dry Sephadex G-200 and used as purified HMW-UK.

Table 1 Purification of urokinase[1])

	Total protein (mg)	Total activity (CTA units)	Specific activity (CTA units per mg)	Yield (%)	purification factor	Mol. weight distribution in %[2]) >100000 UK	HMW UK	LMW UK
Native human urine	750.0 ±229.4	17376.0 ±1522.3	29.9 ±9.7	100.0	1.0	5	90	5
Gelatine Sepharose	28.18 ±8.38	3823.8 ±427.4	154.4 ±49.7	23.0 ±3.5	5.4 ±1.0	15	80	5
Agmatine Sepharose	0.09 ±0.01	2254.0 ±1165.0	28512.0 ±3449.0	15.9 ±4.1	1036.0 ±321.1	5	90	5
Gel filtration	0.02 ±0.01	1928.1 ±903.7	101192.0 ±2962.0	10.5 ±4.0	3891.5 ±1081.5	–	100	–

[1]) Values represent means and S.E. of 6 purifications.
[2]) The molecular weight distribution of UK activities of the starting material and of the materials obtained after each purification step was calculated from the areas of lysis developed after 8 hr at 37° C on plasminogen containing fibrin agarose plates overlayered with slab gels as outlined in the methods. Total area of lysis was defined to be 100%.

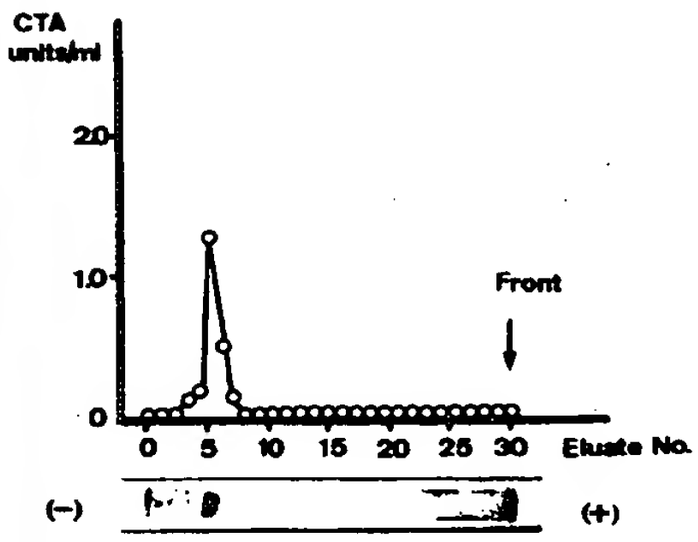

Fig. 5

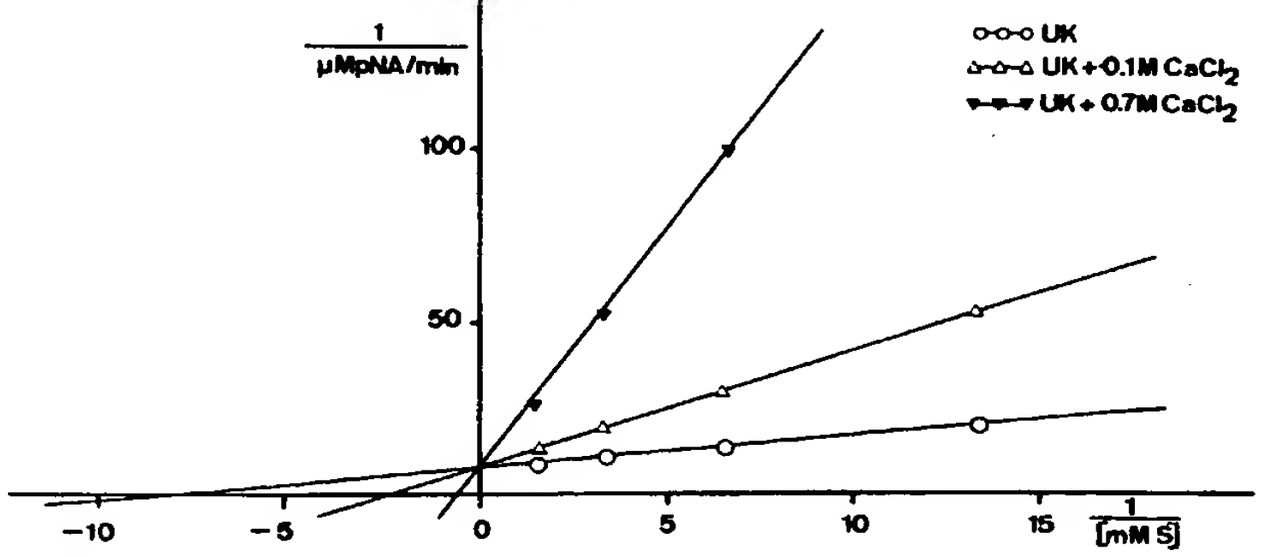

Fig. 6

Fig. 5 Alkaline disc electrophoresis of purified HMW-UK at pH = 8.2. Top: PAA of the eluates of the sliced gel. Bottom: stained replica gel. The anode is to the right.

Fig. 6 Kinetic analysis of the effect of $CaCl_2$ on the amidolytic activity of UK using S-2444 as a substrate. Lineweaver-Burk double reciprocal plot of substrate concentration versus velocity of substrate cleavage.

$11.4 \cdot 10^{-5}$ mol·l^{-1}. Calculation of the maximal velocity resulted in a value of $1.55 \cdot 10^{-10}$ mol·min^{-1}·CTA^{-1}. Addition of $CaCl_2$ at 2 different final concentrations resulted in a decreased cleavage of the pNA-substrate: using different substrate concentrations and analyzing the data by Lineweaver-Burk double reciprocal plot (Fig 6) a common point of intersection at the ordinate for the different $CaCl_2$ concentrations was obtained, while the apparent K_m values increased. Gelatine in different final concentrations in the same assay system led to no significant effects on the cleavage of the substrate.

Using plasminogen as a substrate and monitoring the activation of plasminogen by HMW-UK by measuring plasmin by the synthetic pNA-substrate S-2251 and analyzing the rate of plasmin formation at different substrate and enzyme concentrations by means of a Lineweaver-Burk double reciprocal plot, an apparent K_m value of $1.11 \cdot 10^{-6}$ mol/l and a V value of $0.16 \cdot 10^{-12}$ mol·min^{-1}·CTA^{-1} could be obtained. Addition of different concentrations of gelatine had again no significant effect on the plasminogen activation by the purified UK.

Sucrose density gradient centrifugation of purified UK in 0.01 M Tris.HCl buffer (pH 7.4) either alone or in the presence of gelatine or gelatine and $CaCl_2$ revealed PAA in the fractions obtained after centrifugation corresponding to M_r of 43,000, 43,000, and 47,000, respectively, as determined by the position of marker proteins in the gradient (Fig. 7).

Discussion

The use of native urine as starting material for the isolation of UK is advantageous because concentration or precipitation procedures of the native material as used for preparation of commercially available urokinases may result in alterations of the enzyme and may cause losses of activity. However, dialysis of the crude urine necessary to obtain complete binding of the enzyme to the gelatine-Sepharose led also to appreciable losses of activity although Tween 80 and gelatine had been added during dialysis to increase PAA recovery. From the dialyzed crude urine UK activity could almost completely be adsorbed to the gelatine-

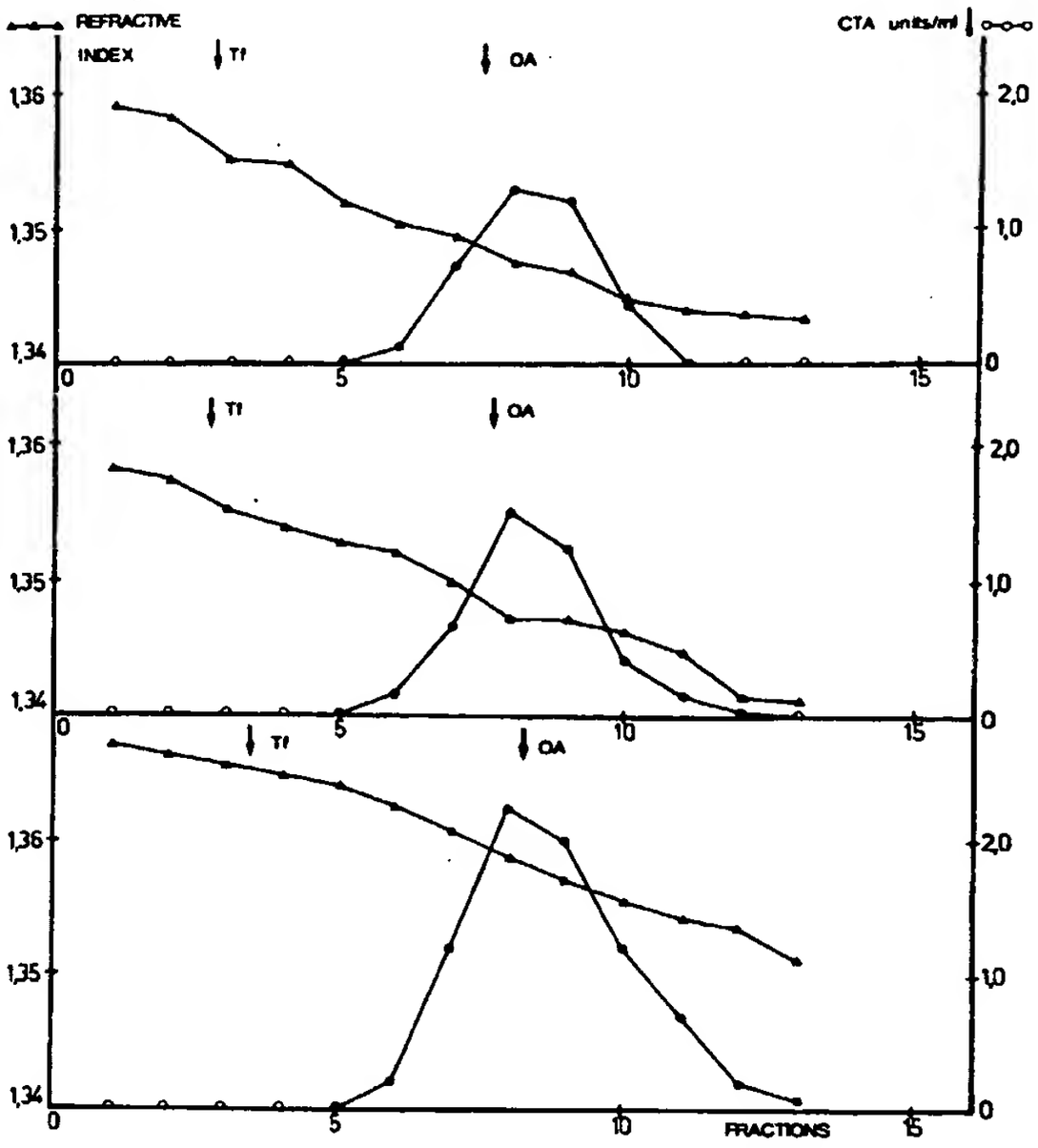

Fig. 7 Sucrose density gradient centrifugation of purified HMW-UK in 5–20% sucrose gradients. Top pannel: UK in 0.01 M Tris.HCl buffer (pH 7.4). Middle pannel: UK preincubated with 0.2% gelatine in 0.01 M Tris.HCl buffer (pH 7.4). Bottom pannel: UK preincubated with 0.2% gelatine in 0.01 M Tris.HCl buffer (pH 7.4) containing 0.5 M $CaCl_2$. Arrows indicate the position of marker proteins in the gradient. (Tf = transferrin; OA = ovalbumin).

Sepharose column and more than 90% of the adsorbed activity could be recovered by a $CaCl_2$ gradient. After the gelatine-Sepharose column step, PAA activity was associated with less than 5% of the protein contained in crude urine. To reduce the time necessary for the gelatine-Sepharose step, batch adsorption and step elution were used without significant changes of recovery or purification factor of the enzyme.

For elution of PAA from the gelatine-Sepharose column calcium was used to increase ionic strength because preliminary experiments with different anions (e.g. sodium, magnesium) showed that calcium eluted PAA in a sharp peak from the column while with other anions PAA was eluted almost during the whole gradient.

This effect might be due to the chaotropic activity of calcium decreasing hydrophobic interactions (31) or to its interactions with collagen (32). In addition, it could be shown that $CaCl_2$ in the concentrations used inhibited the enzyme activity of HMW-UK versus the natural substrate plasminogen and versus a small synthetic pNA-substrate in a way resembling competitive inhibition. This effect might be due to alterations of the substrate binding site of UK by calcium. In order to elucidate the mechanism of interactions between UK and gelatine, sucrose density gradient centrifugation of purified UK with and without addition of gelatine and in the presence and absence of $CaCl_2$ was performed. In all cases, PAA was recovered from the gradient corresponding to a M_r range of 43,000–47,000 indicating no stable direct binding of UK to gelatine. Furthermore, addition of gelatine did not change the enzyme activity of UK versus S-2444 or plasminogen as substrates. Binding of UK to the gelatine column might therefore involve ion exchange or hydrophobic interactions with the gelatine-substituted Sepharose.

The material obtained after the gelatine column was used for further purification by affinity chromatography on agmatine-Sepharose. For this step batch adsorption and step elution were used again. Instead of sodium phosphate, as described originally (13), we used potassium phosphate to avoid formation of salt cristals in the cold. The material obtained after agmatine affinity chromatography was only contaminated by high molecular weight proteins which could completely be removed by gel filtration on Sephadex G-150.

The total recovery of UK activity for the whole purification was 10.5% calculated from crude urine. Losses of activity were mainly due to the first dialysis step but only to a minor extent to the purification itself. As compared to other UK purifications, yields during the purification described are appreciably high. This is most likely due to the short time necessary for the whole procedure which was about 90 hr. Calculated from crude urine the purification factor was about 4,000 whereby affinity chromatography on agmatine-Sepharose was the major purification step.

Physicochemical characterization of the purified HMW-UK revealed a material that could be shown to be homogenous as judged by alkaline disc electrophoresis and that exhibited exclusively an apparent M_r of 54,000 and 56,000 as determined by SDS-slab-PAGE and gel filtration, respectively. The isoelectric point could be determined to be 8.9 (pI range: 8.7 to 9.05). The specific activity could be calculated to be about 100,000 CTA units per mg of protein. All these are characteristics of the HMW-UK which could also be found by different authors using material obtained after different purification procedures (7–9, 17).

Enzyme kinetic analysis of the purified HMW-UK with S-2444 as a substrate gave K_m and V values similar to those reported in the literature (21). Using plasminogen as a substrate the K_m value calculated corresponded to that found by Robbins (29) while the V value was appreciably lower.

To analyze the chain structure of the purified HMW-UK the active center of the enzyme was labelled with ^{3}H-DFP and the labelled material was subjected to SDS-PAGE with and without reduction. Thereby the material was found not to be homogenous but to consist of two different entities, one exhibiting an apparent single chain structure, whereas the other one contained the radiolabel for the active site in a polypeptide chain with a M_r of 31,000. The latter one represents most likely a double chain HMW-UK as seen by Soberano (13) while the former one could be a similar enzyme as the single chain HMW-UK described by Gurewich (33).

Published methods for isolation of UK from native human urine utilized the high affinity of UK to adsorbents (3–6, 9, 10) followed by precipitation (3, 8), ion exchange chromatography (4, 8, 9, 34) and gel filtration (8, 34). Also specific affinity of UK to competitive inhibitors had been included in the purification schemes of prepurified material (11–14). The final products of the purfication schemes exhibited either exclusively the LMW-UK (9) or a mixture of HMW- and LMW-UK (8, 10). However, molecular weight distribution of UK activities in fresh crude urine could be shown to exhibit almost exclusively the HMW-UK accompanied by only small amounts of the LMW-UK and a > 100,000 molecular weight form (15). Therefore the increase in the amount of the LMW-UK during most purification procedures is indicative for alterations of the enzyme during purification. Since in our purification the molecular weight distribution of UK

activities remained similar to that in native urine the enzyme is presumably only slightly altered (Table 1).

The purification scheme described allows rapid isolation of UK to homogeneity without major changes in the molecular characteristics of the enzyme from only 2 liters of urine. Because of the relative low amounts of urine necessary to obtain enough material for characterization it may be possible to purify and characterize UK from individual donors and to correlate changes of UK characteristics with physiological and pathophysiological mechanisms.

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